Opposing actions of Per1 and Cry2 in the Regulation of Per1 Target Gene Expression in the Liver and Kidney

Jacob Richards¹,², Sean All², George Skopis¹, Kit-Yan Cheng¹, Brandy Compton¹, Nitya Srialuri¹, Lisa Stow²†, Lauren A. Jeffers², and Michelle L. Gumz¹,²*

¹Department of Medicine, ²Department of Biochemistry and Molecular Biology
University of Florida, Gainesville, FL 32610

†Current affiliation: SABiosciences, Frederick, MD

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*To whom correspondence should be addressed: Michelle L. Gumz, Department of Medicine, Department of Biochemistry and Molecular Biology, University of Florida, 1600 SW Archer Rd Box 100224. Gainesville, FL 32610, USA, Tel.: (352) 273-6887; fax: (352) 392-5465; E-mail: Michelle.Gumz@medicine.ufl.edu

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ABSTRACT

Mounting evidence suggests that the circadian clock plays an integral role in the regulation of many physiological processes including blood pressure, renal function, and metabolism. The canonical molecular clock functions via activation of circadian target genes by Clock/Bmal1 and repression of Clock/Bmal1 activity by Per1-3 and Cry1/2. However, we have previously shown that Per1 activates genes important for renal sodium reabsorption, which contradicts the canonical role of Per1 as a repressor. Moreover, Per1 KO mice exhibit a lowered blood pressure and heavier body weight phenotype similar to Clock KO mice, and opposite that of Cry1/2 KO mice. Recent work has highlighted the potential role of Per1 in repression of Cry2. Therefore, we postulated that Per1 potentially activates target genes through a Cry2-Clock/Bmal1 dependent mechanism, in which Per1 antagonizes Cry2, preventing its repression of Clock/Bmal1. This hypothesis was tested in vitro and in vivo. The Per1 target genes αENaC and Fxyd5 were identified as Clock targets in mpkCCDc14 cells, a model of the renal cortical collecting duct. We identified PPARα and DEC1 as novel Per1 targets in the mouse hepatocyte cell line, AML12 and in the liver in vivo. Per1 knockdown resulted in upregulation of Cry2 in vitro and this result was confirmed in vivo in mice with reduced expression of Per1. Importantly, siRNA-mediated knockdown of Cry2 and Per1 demonstrated opposing actions for Cry2 and Per1 on Per1 target genes, supporting the potential Cry2-Clock/Bmal1 dependent mechanism underlying Per1 action in the liver and kidney.
INTRODUCTION

The circadian clock is an important regulator of many physiological functions including blood pressure, metabolism, and endocrine, immune, vascular, and renal function (1, 6, 30, 40, 43, 44, 49). The canonical circadian clock molecular mechanism, known as the transcription translational oscillating (TTO) loop, involves four core proteins which interact with one another to regulate transcription of circadian target genes (3). The four major canonical clock proteins are: Bmal1, Clock, Period (Per) (homologs 1-3), and Cryptochrome (Cry) (homologs 1-2). Clock and Bmal1 form a heterodimer, interacting with E-Box response elements in circadian target genes (2, 13). Two of these genes include Per and Cry. Per and Cry are thought to interact and then repress the transcriptional activity of Clock/Bmal1. In addition to transcriptional regulation, the circadian clock also undergoes post-translational modifications through the phosphorylation of the Per proteins by casein kinase 1 isoforms $\delta/\epsilon$ (CK1$\delta/\epsilon$). Phosphorylation by CK1$\delta/\epsilon$ allows Per1 entry into the nucleus (28, 42).

It was initially assumed that Per1 and Per2 shared similar responsibilities in the TTO loop, but recent work by our lab and others has begun to shed light on the possibility that Per1 and Per2 have distinct functions. Recent work in vivo and in vitro has shown that Per2 bridges the Clock/Bmal1 complex to Cry, allowing Cry to repress Clock/Bmal1 transcriptional activity in liver (8). This complex did not appear to require Per1. Studies using Per1/Cry2 knockout (KO) mice showed that Per1 represses Cry2 (36, 37). Another study showed that this repression occurred via a transcriptional mechanism, which involved promotion of transcriptional termination and prevented transcriptional re-initiation of the Cry2 gene (39). Our work has recently highlighted the role of Per1 in the basal and aldosterone-mediated regulation of $\alpha$ENaC, a subunit of the renal epithelial sodium channel (ENaC) (17-19, 50). Per1 also coordinately
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regulates multiple genes involved in sodium reabsorption in the kidney (50). These include the positive regulation of Fxyd5, a positive regulator of the Na,K-ATPase (31) and the negative regulation of Endothelin-1 (ET-1), which is a powerful inhibitor of epithelial sodium channel (ENaC) activity via an Endothelin-B receptor and nitric oxide dependent mechanism (7). This coordinate regulation predicts that loss of Per1 should result in renal sodium wasting, leading to decreased plasma volume and subsequent decreased blood pressure (BP). Indeed, we have recently shown that Per1 KO mice have lower BP compared to wild type (WT) controls (50). Cry1/2 KO mice exhibited salt sensitive hypertension due to an up-regulation of aldosterone production (14). Clock KO mice, however, were relatively hypotensive and exhibited dysregulated sodium excretion and mild diabetes insipidus (34, 62). Cry1/2 KO mice are lean (24), while Clock (Δ19) mutant animals, in which the Clock/Bmal1 heterodimer cannot bind DNA, are obese (53). Interestingly, even though Per1 is assumed to be a repressor, the Per1 KO BP phenotype appears to more closely resemble that of the Clock KO, whereas the Per1 KO BP phenotype appears to be opposite that of the Cry1/2 KO.

The goal of the present study was to investigate the mechanism underlying our previously observed non-canonical actions of Per1 in the positive regulation of gene expression in the kidney. In light of the recent work highlighting the possible roles of Per1 in the transcriptional repression of Cry2, we postulated that a potential mechanism for Per1 activation of renal sodium handling genes is through a Cry2-Clock/Bmal1 dependent mechanism. Specifically, and consistent with the findings of others (33, 36, 37, 39), we hypothesized that Per1 would repress Cry2, preventing its repression of Clock/Bmal1 (Figure 1). However, as most of these studies have been performed in other tissue systems it was important to test our hypothesis in the kidney and to compare those results to the more thoroughly characterized liver peripheral clock. Much
of the mechanism for circadian action has been identified in liver. Therefore, in this present
study we tested the hypothesis that Per1 action on target genes occurs through a Cry2-
Clock/Bmal1 dependent mechanism in both liver and kidney. Our results indicate that Cry2 is
upregulated in the absence of Per1, and that Per1 and Cry2 appear to mediate opposing action on
target gene expression. These findings may help explain the mechanism by which Per1 appears
to activate gene expression.

MATERIALS AND METHODS

Cell Culture. Alain Vandewalle (INSERM, Paris, France) kindly provided the mpkCCDc14 cells
(5). AML12 cells were a gift from Yuri Sautin (University of Florida, Gainesville, FL) (46).
mpkCCDc14 cells were maintained in DMEM-F12 plus 10% FBS and 50 μg/ml gentamicin.
AML12 cells were maintained in DMEM-F12 plus 10% heat inactivated FBS, 5 μg/ml insulin, 5
ng/ml sodium selenite, 5 μg/ml apo-transferrin, 100 U/ml penicillin, 100 μg/ml streptomycin,
and 40 ng/ml dexamethasone.

For RNA silencing experiments, Non-Target-2 and SMARTpool siRNA for CK1δ,
CK1ε, Clock, and Cry2 were purchased from Dharmacon (Cat# D-001206-14-20, L-044377-00-
0005, L-040108-00-0005, L-040484-01-0005, and L-040486-00-0005). A previously
characterized individual siRNA for Per1 was purchased from Dharmacon (J-040487-08-0005)
(19). siRNA were used according to the manufacturer’s instructions. The mpkCCDc14 and
AML12 cells were transfected using Dharmafect 4, as described previously (17, 19, 42).

Casein Kinase 1δ/ε inhibitor experiments were performed as described previously (42).
Twenty-four hours after cells reached 100% confluence, cells were treated with 10μM PF670462
(Santa Cruz) or vehicle (DMSO) for 72 hours. Final DMSO concentration in both vehicle and
inhibitor-treated cells was 0.1%.
Generation of Per1 shRNA in mpkCCD$_{c14}$ cells. SMARTvector 2.0 Lentiviral particles containing Per1 shRNA or Non-Target shRNA were purchased from Dharmacon and used per the manufacturer’s instructions. The vector contained a puromycin resistance cassette and GFP for selection. Lentivirus particles either containing Non-Target or Per1 shRNA were transduced into 40,000 mpkCCD$_{c14}$ cells. Cells that were successfully transduced were first selected using puromycin. Cells were then further assessed using FACS analysis through the Interdisciplinary Center for Biotechnology Research at the University of Florida (http://www.biotech.ufl.edu/) to determine percent positive GFP. Cells that were both puromycin resistant and GFP positive were then plated and individual clones were separated and grown to confluence. Per1 mRNA knockdown was assessed by QPCR. Clones that had sufficient knockdown (greater than 75%) were used for further experiments.

Animals. All animal-use protocols were approved by the University of Florida and North Florida/South Georgia Veterans Administration Institutional Animal Care and Use Committee in accordance with the National Institutes of Health, Guide for the Care and Use of Laboratory Animals. Per1 KO and WT mice (129/sv) were originally provided by Dr. David Weaver (University of Massachusetts (4)). WT and Per1 heterozygote (het) mice were bred in house by UF Animal Care Services Staff. Animals were maintained on a normal 12 hour light:dark cycle. Mice were fed normal lab chow and given free access to water. At midnight, mice were anesthetized and tissues were collected and snap frozen in liquid nitrogen. Kidneys were later dissected and cortex removed for protein or RNA isolation.

RNA Isolation and QPCR. Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions. RNA (10 µg) was treated with DNA-free DNaseI (Ambion). DNaseI-treated RNA (2 µg) samples were used as template for reverse transcription with High
Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNAs (20 ng) were then used as template in quantitative real-time PCR (QPCR) reactions (Applied Biosystems) to evaluate changes in Per1, Clock, Bmal1, Cry2, αENaC, ET-1, Fxyd5, PPARα, and DEC1 mRNA levels. Cycle threshold (Ct) values were normalized against β-actin and relative quantification was performed using the ΔΔCt method (29). Fold change values were calculated as the change in mRNA expression levels relative to the control. TaqMan primer/probe sets were purchased from Applied Biosystems.

Protein Isolation and Western Blot Analysis. Nuclear and cytosolic extracts were isolated using the NE-PER kit (Pierce) according to the manufacturer's instructions. Whole tissue extracts were isolated using IP Lysis Buffer (Pierce) according to the manufacturer’s instructions. Protein concentrations were then quantified by BCA assay (Pierce). Proteins were separated on a 4-20% Tris-HCl Ready Gel (BioRad), and transferred to a PVDF membrane. The membrane was blocked with 2% non-fat dry milk in TBS-S (TBS plus 0.05% Rodeo™ Saddle Soap) (USB) and incubated overnight at 4°C with anti-Clock (1:1000)(Pierce), anti-Per1(1:500)(Pierce), anti-Bmal1 (1:500)(Pierce), anti-Cry2 (1:500)(Thermo Scientific), anti-Per2 (1:500)(Santa Cruz), anti-PPARα (Santa Cruz), anti-DEC1 (Santa Cruz), or anti-β-actin (1:500)(Santa Cruz) antibodies. β-Actin was used as a loading control. The membrane was washed with 2% non-fat dry milk in TBS-S for 15 minutes and then incubated with horseradish peroxidase conjugate anti-rabbit secondary antibody and incubated in 2% non-fat dry milk in TBS-S for 1 hour at 4 °C. After incubation, the blot was washed with TBS-S for 15 minutes. Detection was performed using Novex® ECL Chemiluminescent Substrate reagents (Invitrogen). Densitometry was performed using ImageJ (rsbweb.nih.gov/ij).
Co-Immunoprecipitation. Co-immunoprecipitations were performed using the Pierce Co-immunoprecipitation kit (Pierce) according to the manufacturer’s instructions. Anti-Per1 antibody columns were created using 40μg of antibody (Thermo Scientific) and 500μg of nuclear extract were loaded onto the column. Nuclear extracts were pre-cleared with control agarose resin (Pierce) per the manufacturer’s instructions.

Statistical Analysis. Student's unpaired t-test (Graphpad) was used to compare two data sets. Data are presented as the mean±standard error of the mean (SEM). P values less than 0.05 were considered significant.

RESULTS

Conserved Canonical Clock Interactions are Preserved in Kidney Cortex and Liver. As mentioned above, the core circadian clock complex consists of four major circadian proteins (Per, Cry, Clock, and Bmal1) that have been shown to interact in mouse liver and mouse embryonic fibroblasts (8). Though the existence of circadian rhythms in the kidney has been well established (reviewed in (49)), the presence of the core circadian complex has not been confirmed. Therefore we performed co-immunoprecipitations in kidney cortex and liver to test which of the core clock proteins associated with Per1 at midnight. As mice are nocturnal, midnight is the peak of their active phase, when circadian proteins are activated (2, 25, 27). We and others have observed the existence and action of a smaller nuclear Per1 fragment (~50kDa)(9, 17, 42, 50); this nuclear form of Per1 interacts with E-boxes from the αENaC and ET-1 promoters (17, 39, 46). As shown, Per1 associates with Clock, Bmal1, Cry2, and Per2 in nuclear extracts derived from the renal cortex and the liver of wild type mice euthanized at midnight (Figure 2). It is not surprising that Per1 associates with itself as it has been shown
previously that both Per1 and Per2 can homodimerize (21, 26, 61). Two recent studies have
previously demonstrated the interaction of these proteins in mouse liver (8, 25).

*αENaC and Fxyd5 are Per1 and Clock Target Genes.* We have previously shown that
αENaC, Fxyd5, and Endothelin-1 are Per1 target genes (17, 18, 50). To determine if they were
also Clock target genes, mpkCCD_c14 cells, a model of the renal cortical collecting duct (5), were
treated with Per1 siRNA, Clock siRNA, or a combination of Per1 and Clock siRNA (Figure 3).

As expected, mRNA expression of both Per1 and Clock was decreased with the respective
siRNAs (Figure 3A and B). Interestingly, in both cases, Per1 siRNA resulted in reduced Clock
mRNA and Clock siRNA resulted in reduced Per1 mRNA. It is not surprising that Clock siRNA
positively affects Per1 levels, since Clock/Bmal1 activity is known to increase Per1 expression
(2, 22, 58). As we have shown previously (17, 50), both αENaC and Fxyd5 were significantly
decreased with Per1 siRNA (Figure 3C and D). Fxyd5 and αENaC were also affected by Clock
siRNA. Combination treatment resulted in knockdown that was not statistically different from
individual treatments. Taken together these data suggest that both Fyxd5 and αENaC are Clock
target genes. Interestingly, ET-1 was not significantly affected by Clock siRNA (Figure 3E),
suggesting a potential Clock-independent mechanism for the repression of ET-1 by Per1.

*Cry2 Protein is Upregulated Following Per1 Knockdown in mpkCCD_c14 Cells.* As
described above, αENaC and Fxyd5 appear to be target genes of both Per1 and Clock. Our
hypothesis is that this mechanism of regulation is Cry2-dependent. Previous work has shown that
Per1 suppresses Cry2 transcriptionally (15, 36, 37); these findings predict that Per1 knockdown
should result in upregulation of Cry2. To test this in mpkCCD_c14 cells, Per1 was stably knocked
down using lentiviral particles containing Per1 or Non-Target shRNA. Cells were then selected
using puromycin resistance and GFP cell sorting. Remaining cells were plated, clones isolated,
grown to confluence, and selected based on reduction of Per1 and αENaC mRNA expression determined by QPCR (data not shown). Per1 protein levels were assessed in Per1 shRNA clones 3, 7, and 10 by western blot. As mentioned above, the existence and action of a smaller nuclear Per1 fragment (~50kDa) has been observed by our lab and others (9, 17, 42, 50); this nuclear form of Per1 interacts with E-boxes from the αENaC and ET-1 promoters (17, 39, 46). As shown, nuclear Per1 protein levels were decreased in all three clones (Figure 4A, quantified in Figure 4B). Protein levels of Clock, Bmal1, and Cry2 were also assessed by western blot (Figure 4A). Consistent with our hypothesis, western blot analysis showed that nuclear Cry2 levels were increased in all three Per1 shRNA clones (Figure 4B). Nuclear Clock and Bmal1 protein levels did not appear to be altered.

Identification of PPARα and DEC1 as Per1 Target Genes in AML12 Cells and in Liver.

Peroxisome proliferator-activated receptor alpha (PPARα) is a lipid sensor that is activated by excess fatty acids and regulates lipoprotein synthesis and gluconeogenesis in the liver (41). Once activated, PPARα induces expression of downstream genes that metabolize fatty acids. It has been recently shown to be a circadian target gene (35, 55-57). Differentiated embryo chondrocyte 1 (DEC1), also known as basic helix-loop-helix protein 2, Sharp-2, or STRA13, is a basic-helix-loop-helix transcriptional factor that binds to E-box response elements. DEC1 has been implicated in circadian control by competing for binding to E-boxes with the Clock-Bmal1 complex (23). It has also been implicated in regulation of adipogenesis (38), regulation of metabolite-sensing nuclear receptors (10), chondrogenesis (47), neurogenesis (45), T-lymphocyte activation (51), and cell growth arrest (52). DEC1 has also been identified as a circadian target gene (54).
We tested if PPAR\(\alpha\) and DEC1 were Per1 targets in the mouse hepatocyte cell line, AML12 (46). AML12 cells were treated with Per1 siRNA, Clock siRNA or a combination of both (Figure 5). As was observed in the mpkCCD\(_{c14}\) cells, both Per1 and Clock were decreased following treatment of AML12 cells with the respective siRNAs (Figure 5A and B). Per1 siRNA resulted in decreased Clock expression and Clock siRNA resulted in decreased Per1 expression. This result was also observed in the mpkCCD\(_{c14}\) cells, as described above. PPAR\(\alpha\) and DEC1 mRNA were both decreased following Per1 knockdown (Figure 5C and D), suggesting that they are Per1 target genes. Both were decreased with Clock siRNA confirming that they are Clock target genes as well. A further decrease in expression was not observed following treatment with the combination of Per1 and Clock siRNA.

To determine if PPAR\(\alpha\) and DEC1 are Per1 targets in vivo, expression levels were determined in available WT and Per1 het tissue. First, Per1 protein expression was measured in whole tissue extracts from liver and kidney cortex (Figure 6 A and B). As expected, Per1 het mice exhibited approximately 50% less Per1 in both the kidney cortex (Figure 6A) and the liver (Figure 6B) compared to wild type animals (It should be noted that circadian behavioral analysis has not been performed in Per1 het mice). Next, confirming our in vitro findings, mRNA expression of both PPAR\(\alpha\) and DEC1 was measured and found to be decreased in Per1 het mice compared to WT (Figure 7A and B). Consistent with our mRNA data, reduced Per1 levels resulted in a modest decrease in PPAR\(\alpha\) and DEC1 nuclear protein levels (Figure 7C and D).

Per1 must be phosphorylated by CK1\(\delta/\varepsilon\) in order to enter the nucleus (28). We recently showed that pharmacological inhibition of CK1\(\delta/\varepsilon\) with PF670462 in mpkCCD\(_{c14}\) cells recapitulated the effects of Per1 knockdown, including decreased \(\alpha\)ENaC mRNA and protein expression; treatment with this drug decreased ENaC channel activity as well (42). Therefore, in
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order to further confirm the effects of Per1 on PPARα and DEC1, AML12 cells were treated with PF670462 (8). As expected, CK1δ/ε inhibition resulted in a decrease in nuclear Per1 abundance (Figure 8A, quantified in Figure 8B). CK1δ/ε inhibition resulted in decreased PPARα and DEC1 mRNA (Figure 8C and D). To further confirm this effect, AML12 cells were transfected with CK1δ/ε siRNA (Figure 8E and F). PPARα and DEC1 were decreased following CK1δ/ε knockdown (Figure 8G and H). Taken together, these data demonstrate that Per1 knockdown, either in vitro with siRNA, indirectly with CK1δ/ε inhibition, or in vivo, resulted in decreased PPARα and DEC1 mRNA.

Cry2 Protein is Upregulated Following Pharmacological Inhibition of Per1 Nuclear Entry in AML12 Cells. To test the effect of Per1 knockdown on Cry2 protein levels in AML12 cells, cells were treated with CK1δ/ε inhibitor (PF670462) to reduce nuclear Per1, and protein levels were assessed by western blot (Figure 9). Cry2 protein levels were significantly elevated in both the cytosol and nucleus following CK1δ/ε inhibition (Figure 9A). This result was quantified (Figure 9B), demonstrating that indirect inhibition of Per1 resulted in significant increases in cytosolic and nuclear Cry2 levels. Thus, in both mpkCCDc14 and AML12 cells, CK1δ/ε mediated-Per1 knockdown resulted in increased Cry2 levels, a result that is consistent with suppression of Cry2 by Per1.

Per1 Suppresses Cry2 in vivo in Kidney Cortex and Liver. To determine if Per1 suppresses Cry2 in vivo, nuclear Cry2 protein levels were measured by western blot analysis of nuclear fractions from kidney cortex and liver of WT and Per1 het mice euthanized at midnight (Figure 10). In kidney cortex and liver, nuclear Cry2 levels were significantly elevated when compared to WT (Figure 10A.) Densitometry analysis indicated that nuclear Cry2 levels were increased two fold in both kidney cortex and in liver (Figure 10B). Nuclear Clock and Bmal1
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protein levels were not significantly altered in Per1 het compared to WT (Figure 10A and B). To
determine if suppression of Cry2 by Per1 occurred at the level of mRNA, Cry2 mRNA levels
were measured in kidney cortex and liver by QPCR. Cry2 mRNA levels were significantly
upregulated in both tissues, supporting the hypothesis that suppression of Cry2 by Per1 is at least
partially dependent on regulation of mRNA expression (Figure 10C).

Per1 and Cry2 Mediate Opposing Actions on Per1 Target Genes in mpkCCDc14 and
AML12 Cells. We hypothesized that Per1 action is mediated through a Cry2-Clock/Bmal1-
dependent mechanism in which Per1 represses Cry2, preventing Cry2 from repressing
Clock/Bmal1 action (Figure 1). This hypothesis predicts that Per1 and Cry2 may mediate
opposing actions on Per1 target gene expression. To test this hypothesis, mpkCCDc14 and
AML12 cells were treated with Per1 siRNA, Cry2 siRNA, or Clock siRNA or a combination of
either Per1 and Cry2 siRNA or Clock and Cry2 siRNA. The effect of these siRNAs on Per1,
Clock and Cry2 was assessed using QPCR (Figure 11). As expected, Per1 mRNA levels in
mpkCCDc14 and AML12 cells were decreased with either Per1 or Clock siRNA. Per1 mRNA
levels were increased with Cry2 siRNA. Per1 levels were decreased with combination treatment
of Cry2 and Per1 siRNA. Interestingly, a combination of Clock and Cry2 siRNA resulted in
normalization of Per1 mRNA expression to control values in both mpkCCDc14 and AML12 cells
(Figures 11A and 10B). Clock mRNA levels in mpkCCDc14 and AML12 cells decreased with
either Per1 siRNA or Clock siRNA (Figures 11C and 11D), as shown previously (see Figures 3
and 5). Cry2 siRNA had no effect on Clock mRNA levels. The combination of Per1 siRNA and
Clock siRNA resulted in decreased Clock mRNA expression. A combination of siRNA against
Clock and Cry2 also resulted in decreased Clock mRNA expression in both cell types (Figures
11C and 11D). In both cell lines, treatment with Per1 siRNA resulted in increased Cry2 mRNA
levels (Figures 11E and 11F), supporting the hypothesis that Per1-mediated suppression of Cry2 may be mRNA dependent. Cry2 siRNA, Clock siRNA, or the combination of Clock and Cry2 siRNAs resulted in decreased Cry2 expression in mpkCCDc14 and AML12 cells (Figures 11E and 11F).

We next assessed the effect of Per1, Clock, and Cry2 action on Per1 target gene expression in mpkCCDc14 cells (Figure 12). As we have shown previously, αENaC mRNA expression decreased with Per1 or Clock knockdown in mpkCCDc14 cells. Importantly, αENaC mRNA levels were increased with Cry2 siRNA-mediated knockdown, suggesting a role for Cry2 in repression of this Per1 target gene. The combination of Per1 and Cry2 siRNA returned expression of αENaC to control levels, supporting our hypothesis that Per1 and Cry2 mediate opposing actions on αENaC gene expression. A combination of Clock and Cry2 siRNA also resulted in control levels of αENaC gene expression (Figure 12A). These effects were observed for Fxyd5 mRNA levels as well (Figure 12B). ET-1, which did not appear to be a Clock target gene, was not affected by Cry2, Clock, or a combination of Clock and Cry2 siRNA, highlighting the potential Clock/Bmal1 independent effect of Per1 on ET-1 (Figure 12C). Per1 or Per1 and Cry2 siRNA resulted in similar increases of ET-1 gene expression (Figure 12C).

Next, we investigated the effect of Per1, Clock, and Cry2 action on Per1 target gene expression in AML12 cells. The effects on αENaC and Fxyd5 expression were mirrored in the AML12 cells for the target genes PPARα and DEC1 (Figure 13). As shown above (see Figure 5), PPARα mRNA expression decreased with Per1 or Clock knockdown (Figure 13A). In contrast, PPARα mRNA levels were increased with Cry2 siRNA-mediated knockdown. The combination of Per1 and Cry2 siRNA returned expression of PPARα to control levels, supporting our hypothesis that Per1 and Cry2 mediate opposing actions on PPARα gene expression as well. A
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combination of Clock and Cry2 siRNA also resulted in control levels of PPARα gene expression (Figure 13A). These effects were observed for DEC1 mRNA levels as well (Figure 13B). Taken together, the results from both mpkCCD_{c14} and AML12 cells demonstrate that Per1 and Cry2 mediate opposing actions on Per1 target genes. These data are consistent with our proposed mechanism of a Cry2-Clock/Bmal1 dependent mechanism for Per1 action in liver and kidney.

Per1 het Mice Weigh more than WT Controls. As shown above, Per1 positively regulates mRNA expression of PPARα. PPARα is critical for metabolic control; PPARα KO mice display increased body weight, hyperlipidemia, decreased serum insulin, and increased inflammation in adipose tissue (60). Per1/2/3 triple KO mice gain significantly more weight on normal or high fat diet than WT controls (12), but such a phenotype has not been assessed for Per1 het mice. To compare body weight between Per1 het and WT, weight measurements were made in two independent sets of age-matched (16 or 28 weeks) WT and Per1 het mice. Indeed, Per1 het mice weigh significantly more than WT controls at both 16 and 28 weeks (Figure 14), a result that is consistent with a role for Per1 in the regulation of PPARα, but likely involves multiple signaling pathways. Importantly, the heavier body weight phenotype of Per1 het provides further support for the hypothesis that Per1 and Cry2 mediate opposing action since Cry1/2 KO exhibit a lean body weight (24). Thus, the blood pressure (50) and body weight phenotype resulting from reduced Per1 expression in vivo resembles that of the Clock KO and is opposite that of the Cry1/2 KO.

DISCUSSION

The goal of this study was to investigate the mechanism underlying our previously observed non-canonical actions of Per1 in the positive regulation of gene expression in the
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kidney. We tested if Per1 action in the liver and kidney was due to a Cry2-Clock/Bmal1 dependent mechanism, in which Per1 suppresses Cry2, preventing Cry2 from suppressing Clock/Bmal1 action (Figure 1). In support of this hypothesis, we found that Per1 target genes in mpkCCDc14 were also Clock target genes. We identified two new Per1 target genes in AML12 cells and in liver, PPARα and DEC1. We confirmed the existence of the canonical clock complex in the kidney in vivo. Both in vitro and in vivo, Per1 knockdown resulted in upregulation of Cry2 at the mRNA and protein level, demonstrating a potential role for Per1 in Cry2 suppression. Importantly, we showed that Per1 and Cry2 mediate opposing actions on Per1 target genes in the liver and kidney.

Our results provide evidence for a Cry2-dependent mechanism underlying Per1 action on target genes. Our data is consistent with what has been observed using behavioral outputs for the role of Per1 in the suppression of Cry2 (36, 37). In these studies, it was demonstrated that Per1/Cry2 double KO mice exhibited normalization of the behavioral phenotype of each single KO, as determined by actogram analysis. This is consistent with our observations that knockdown of both Per1 and Cry2 resulted in normalization of Per1 target gene expression in mpkCCDc14 and AML12 cells. As mentioned above, we have previously shown that Per1 KO mice have significantly lower BP than WT controls (50). This phenotype is opposite that of the Cry1/2 double KOs that exhibit salt sensitive hypertension (14) and similar to that of Clock KO mice, which also display a relatively hypotensive phenotype (62). Furthermore, Cry1/2 KO mice exhibit a lean body weight phenotype (24) whereas Clock (Δ19) mutant mice are obese (53); the results of the current study provide evidence that Per1 het mice, similar to Clock KO mice, exhibit a heavier body weight phenotype. These physiological findings are consistent with our observation that Per1 and Cry2 exert opposing action on target gene regulation. However, the
present study focuses on the opposing actions of Per1 and Cry2, and our results do not preclude
the involvement of Per2 and Cry1 in the regulation of circadian target genes.

Interestingly, repression of ET-1 by Per1 appears to be independent of Bmal1/Clock or
Cry2. ET-1 is a powerful inhibitor of ENaC and thus behaves as a natriuretic hormone in the
renal collecting duct (7). Outside of the kidney, however, ET-1 acts as a potent vasoconstrictor
(59). The tissue specific mechanisms for the actions of Per1 on ET-1 are unknown and further
investigation is required to define the mechanism by which Per1 regulates ET-1 expression in the
kidney.

We have identified two new Per1 targets in the liver, PPARα and DEC1. PPARα is
involved in fatty acid utilization and metabolism. We show that Per1 het mice weigh more than
age-matched WT controls at 16 and 28 weeks, a result that is consistent with the work of others.
Per1/2/3 triple KO mice gain significantly more weight on normal or high fat diet than WT
controls (12). Clock (Δ19) mutant animals, in which the Clock/Bmal1 heterodimer cannot bind
DNA, exhibited metabolic syndrome with hyperlipidemia and obesity (53). Bmal1 KO mice are
obese and exhibit hyperlipidemia (20). In support of the opposing actions of Per1 and Cry2,
Cry1/2 double KO mice are leaner than WT controls (24). Both Clock KO and Bmal1 KO mice
are diabetic with reduced plasma insulin levels (32). As mentioned above, PPARα KO mice
display increased body weight, hyperlipidemia, decreased plasma insulin, and increased adipose
inflammation (60). It will be interesting to determine if downregulation of PPARα in the
circadian mutant animals could be a partial reason for the obesity and hypoinsulinemia observed
in the Clock KO and Bmal1 KO animals. A recent study demonstrated that mice that were fed a
high fat diet during the evening had altered circadian clock gene expression and PPARα
expression. This led to modulation of expression of the PPARα target genes, cholesterol-7-
alpha-hydroxylase and diglyceride acyltransferase (56). These findings demonstrate that circadian clock modulation of PPARα expression leads to altered expression of PPARα target genes.

DEC1 has been implicated in adipogenesis (38). To the best of our knowledge, a metabolic phenotype has not been reported for DEC1 KO mice. DEC1 has been implicated in circadian control by competing for binding to E-boxes with the Clock-Bmal1 complex (23). It is important to note that due to the implications of DEC1 in the regulation of the circadian clock, it would be difficult to ascertain differences between the effects of the circadian proteins versus DEC1 in the causality of a metabolic phenotype.

It is highly likely that Per1, as part of the circadian clock mechanism, regulates a multitude of other genes in the liver that pertain to metabolism. Studies have shown that over 25% of metabolites display circadian oscillations in mice and humans (11, 16). Around 10% of genes in the liver, including many rate-limiting metabolic enzymes, are rhythmically expressed (48). A recent global ChIP seq study looking at DNA binding for Per1, Per2, Cry1, Cry2, Clock and Bmal1, showed high enrichment of these proteins at multiple genes involved in common metabolic pathways (25).

In summary, the results of the present study support the hypothesis that Per1 target gene activation potentially occurs through a Cry2-Clock/Bmal1 dependent mechanism in which Per1 action de-represses Clock/Bmal1 (Figure 1). These data are consistent with previous studies demonstrating that Per1 transcriptionally suppresses Cry2 (33, 36, 37, 39). Taken together, our observations lend support to a hypothetical model in which Per1 action on target genes may occur via a potential Cry2-Clock/Bmal1 dependent mechanism.

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FIGURE LEGENDS

Figure 1. Hypothetical model of Per1 action in kidney and liver. Hypothetical model in which Per1 represses Cry2, preventing its repression of Clock/Bmal1, resulting in activation of Per1 target genes in the kidney (αENaC and Fxyd5) and liver (PPARα and DEC1).

Figure 2. Canonical clock interactions exist in kidney cortex. Dissected cortex and whole liver were harvested from wild type 129/sv mice euthanized at midnight. Nuclear extracts were collected and Co-immunoprecipitations were performed using anti-Per1 antibody (Thermo Scientific) with respective positive and negative controls. Positive control consisted of protein input. Negative controls consisted of replacing anti-Per1 resin with inactive cross-linked resin (Pierce). Data are representative of three independent experiments. Western blot analysis was performed using anti-Per1 (Thermo Scientific), anti-Cry2 (Thermo Scientific) anti-Clock (Thermo Scientific), anti-Bmal1 (Thermo Scientific), and anti-Per2 (Santa Cruz) antibodies.

Figure 3. αENaC and Fxyd5 are Clock targets, while ET-1 is “Clock-independent”. mpkCCD_{c14} cells were treated with Non-Target, Per1, or Clock siRNA or a combination of Per1 and Clock siRNA for 48 hours. QPCR was used to evaluate changes in Per1 (Panel A), Clock (Panel B), αENaC (Panel C), Fxyd5 (Panel D), or ET-1 (Panel E) gene expression in Per1, Clock, or Clock/Per1 siRNA treated cells versus Non-Target siRNA control. *p<0.05, **p<0.01, *** p<0.001, n=4. Values are represented as the mean ± SEM.

Figure 4. Per1 knockdown results in upregulation of Cry2 in vitro in mpkCCD_{c14} cells. Lentiviral particles either containing Non-Target or Per1 shRNA were transfected into 40,000 mpkCCD_{c14} cells, puromycin and GFP selected, and, ten individual clones were randomly
selected, separated, and grown to confluence. Western blot analysis was performed using anti-
Per1, anti-Bmal1, anti-Clock, anti-Cry2 antibodies. Anti-β-actin was used as a loading control.
Densitometry analysis was used to quantitate the level of Per1 and Cry2 in panel A. *p<0.05
relative to Non-Target shRNA, n=3. Values are represented as the mean ± SEM.

**Figure 5. PPARα and DEC1 are Per1 and Clock target genes.** AML12 cells were treated
with Non-Target, Per1, Clock siRNA or a combination of Per1 and Clock siRNA for 48 hours.
QPCR was used to evaluate changes in Per1 (Panel A), Clock (Panel B), PPARα (Panel C),
DEC1 (Panel D) expression in Per1, Clock, or Clock/Per1 siRNA treated cells versus non-target
siRNA control, n=4 *p<0.05, **p<0.01, *** p<0.001. Values are represented as the mean ±
SEM.

**Figure 6. Per1 het mice exhibit reduced levels of Per1 protein.** Whole tissue extracts were
isolated from kidney cortex (Panel A) and liver (Panel B) from WT 129/sv and Per1 het mice
euthanized at midnight. Western blot analysis was performed using an anti-Per1 antibody to
evaluate Per1 protein expression with β-Actin as a loading control. Data are representative of
four independent experiments. Densitometry analysis was used to quantitate expression (lower
panels), n=4 *p<0.05, Values are represented as the mean ± SEM.

**Figure 7. Reduced Per1 levels in vivo results in decreased levels of PPARα and DEC1.**
Whole livers were harvested from WT 129/sv and Per1 het mice euthanized at midnight; nuclear
extracts and RNA were collected. A-B. Changes in PPARα and DEC1 mRNA were determined
by QPCR (Panel A and B) *p<0.05, n=4. C-D. Western blot analysis was performed using anti-
PPARα, or anti-DEC1 to evaluate changes in PPARα (C) and DEC1 (D) protein expression with
β-Actin as a loading control. Data are representative of four independent experiments. Values are
represented as the mean ± SEM.
Figure 8. Inhibition of CK1δ/ε reduces PPARα and DEC1 expression. A. Nuclear and cytosolic extracts were collected from AML12 cells treated with 10μM Casein Kinase 1δ/ε inhibitor PF670462 for 72 hours. A. Western blot analysis was performed using anti-Per1 or anti-β-actin antibodies as a loading control. Data are representative of three independent experiments. B. Densitometry analysis was used to quantitate the level of Per1 in panel A, n=3. C-D. AML12 cells were treated with 10μM Casein Kinase 1δ/ε inhibitor PF670462 for 72 hours. QPCR was used to evaluate changes in PPARα (Panel C), DEC1 (Panel D), gene expression in PF670462-treated cells versus DMSO-treated cells. n=3 E-H AML12 cells were treated with Non-Target or Casein Kinase 1δ and ε siRNA for 48 hours. Real time PCR was used to evaluate changes in CK1δ (Panel E), CK1ε (Panel F), PPARα (Panel G) or DEC1 (Panel H) gene expression in Casein Kinase 1δ/ε siRNA treated cells versus Non-Target. *p<0.05, **p<0.01, *** p<0.001, n=4. Values are represented as the mean ± SEM.

Figure 9. Per1 knockdown results in upregulation of Cry2 in vitro in AML12 cells. A. Nuclear and cytosolic extracts were collected from AML12 cells treated with 10μM Casein Kinase 1δ/ε inhibitor PF670462 for 72 hours. Western blot analysis was performed using anti-Cry2 or anti-β-actin antibodies as a loading control. Data are representative of three independent experiments. B. Densitometry analysis was used to quantitate the level of Cry2 in panel G. *p<0.05 relative to vehicle, †p<0.5 relative to cytosolic, n=3. Values are represented as the mean ± SEM.

Figure 10. Reduced Per1 expression results in upregulation of Cry2 in vivo in mouse kidney cortex and liver. Dissected cortex and whole liver were harvested from WT 129/sv and Per1 het mice euthanized at midnight and nuclear extracts collected. A. Western blot analysis was
performed using anti-Bmal1, anti-Clock, or Anti-Cry2 to evaluate changes in Bmal1, Clock, and Cry2 protein expression with β-Actin as a loading control. Data are representative of five independent experiments. B. Densitometry analysis was used to quantitate the level of Bmal1, Clock, and Cry2 in panel A. n=5. C. QPCR was used to evaluate changes in Cry2 gene expression in WT versus Per1 het mice. n=4. *p<0.05, **p<0.01. Values are represented as the mean ± SEM.

Figure 11. Effect of Per1, Cry2, and Clock on circadian gene expression in mpkCCDc14 and AML12 cells. mpkCCDc14 or AML12 cells were treated with Non-Target, Per1, Cry2, Clock siRNA or a combination of both Per1 and Cry2 or Cry2 and Clock for 48 hours. A-C QPCR was used to evaluate changes in Per1 (Panel A-B), Clock (Panel C-D), Cry2 (Panel E-F), gene expression in mpkCCDc14 and AML12 siRNA treated cells versus Non-Target. p<0.05, **p<0.01, n=3. Values are represented as the mean ± SEM.

Figure 12. Per1 and Cry2 mediate opposing action on Per1 target genes in mpkCCDc14 cells. mpkCCDc14 cells were treated with Non-Target, Per1, Cry2, Clock siRNA or a combination of both Per1 and Cry2 or Cry2 and Clock for 48 hours. QPCR was used to evaluate changes in αENaC (Panel A), Fxyd5 (Panel B), ET-1 (Panel C), gene expression in mpkCCDc14 siRNA treated cells versus Non-Target. p<0.05, **p<0.01, n=3. Values are represented as the mean ± SEM.

Figure 13. Per1 and Cry2 mediate opposing action on Per1 target genes in AML12 cells. AML12 cells were treated with Non-Target, Per1, Cry2, Clock siRNA or a combination of both Per1 and Cry2 or Cry2 and Clock for 48 hours. QPCR was used to evaluate changes in PPARα (Panel A), and DEC1 (Panel B) gene expression in AML12 siRNA treated cells versus Non-Target. p<0.05, **p<0.01, n=3. Values are represented as the mean ± SEM.
Figure 14. Per1 het mice weigh more than WT controls. Body weight measurements of an independent set of 16 (A) and 28 (B) week old WT or Per1 het mice. *p<0.05, n=9-12. Values are represented as the mean ± SEM.
Figure 1

Key:
- **Per1**
- **Cry2**
- **Bmal1**
- **Clock**
- αENaC, Fxyd 5
- PPARα, DEC1

**Kidney**

**Liver**
Figure 4

A

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B

- Relative Per1 Protein Abundance
  - Non-Target shRNA: 1.00 ± 0.10
  - Per1 shRNA Clone 3: 0.50 ± 0.05
  - Per1 shRNA Clone 7: 0.50 ± 0.05
  - Per1 shRNA Clone 10: 0.50 ± 0.05

- Relative Cry2 Protein Abundance
  - Non-Target shRNA: 10.0 ± 2.0
  - Per1 shRNA Clone 3: 10.0 ± 2.0
  - Per1 shRNA Clone 7: 10.0 ± 2.0
  - Per1 shRNA Clone 10: 10.0 ± 2.0

* indicates statistical significance compared to Non-Target shRNA
Figure 6

A  Kidney Cortex

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B  Liver

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**Relative Per1 Protein Abundance**

- **Kidney Cortex**
  - WT: 1.00
  - Per1 het: 0.50

- **Liver**
  - WT: 1.00
  - Per1 het: 0.50